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CELLULAR EVENTS IN THE PROTHORACIC GLANDS AND ECDYSTEROID TITRES DURING THE LAST-LARVAL INSTAR OF *SPODOPTERA LITTORALIS*

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Abstract—Changes in prothoracic gland morphology were correlated to developmental events and ecdysteroid titres (20-hydroxyecdysone equivalents) during the last-larval instar in *Spodoptera littoralis*. After ecdysis to the last-larval instar the haemolymph ecdysteroid titre remained at about 45 ng/ml, when the prothoracic glands appeared quiescent. The first signs of distinct gland activity, indicated by increased cell size and radial channel formation, were observed at about 12 h prior to the cessation of feeding (36 h after the last-larval moult), accompanied by a gradual increase in ecdysteroid titre to 110 ng/ml haemolymph, at the onset of metamorphosis. During this phase ecdysteroid titres remained at a constant level (140–210 ng/ml haemolymph) and prothoracic gland cellular activity was absent for a short period. The construction of pupation cells occurred when haemolymph ecdysteroids titres increased to 700 ng/ml. A rapid increase in ecdysteroids began on the fourth night (1600 ng/ml haemolymph) reaching a maximal level (4000 ng/ml haemolymph) at the beginning of the fourth day. In freshly moulted pupae a relatively high ecdysteroid titre (1100 ng/ml haemolymph) was still observed, although during a decrease to almost negligible levels. The increase in ecdysteroid level during the third and the fourth nights of the last-larval instar was correlated with the period when almost all the prothoracic gland cells showed signs of high activity. Neck-ligation experiments indicated the necessity of head factors for normal metamorphosis up to the second to third day of the instar. The possibility that the prothoracic glands are under prothoracicotropic hormone regulation at these times is discussed.

Key Word Index: *Spodoptera littoralis*, prothoracic gland morphology, ecdysteroid radioimmunoassay, insect development

INTRODUCTION

The orderly sequence of events allowing normal development to proceed is to a large extent effected by hormonal regulation. In insects, 20-hydroxy ecdysone influences a variety of developmental processes throughout the life cycle, notably stimulation of the larval, and larval–pupal ecdyses (see Gilbert *et al.*, 1980). The production of 20-hydroxy ecdysone is the result of a prohormone, ecdysone, being released by the prothoracic glands (King *et al.*, 1974), with subsequent hydroxylation by peripheral tissues (King and Siddall, 1969). Ecdysteroid radioimmunoassay (RIA) studies in a variety of insects indicate that 20-hydroxy ecdysone-mediated events coincide with discrete surges, or elevated hormone levels (Bollenbacher *et al.*, 1981; Sehnal *et al.*, 1981; Lagueux *et al.*, 1979). Thus, synthesis, secretion, and metabolism of 20-hydroxy ecdysone must be developmentally regulated. Positive and negative regulation of hormone synthesis and secretion may be effected by neural control of endocrine gland function, or by feedback effects by the glandular product or other hormones. Initially, to study the regulation of prothoracic gland activity during development of the cotton leafworm *Spodoptera littoralis*, we have correlated specific developmental events during the last-larval instar with changes in the general gland morphology and cytology indicating cellular activity, and changes in haemolymph ecdysteroid titres.

Prothoracic gland structure has already been correlated with known endocrine activity in various holometabolous (McDaniel *et al.*, 1976; Glitho *et al.*, 1979), and hemimetabolous (Smith and Nijhout, 1982; Hirn *et al.*, 1974) insect species. In *Manduca sexta*, the relationship between ultrastructural changes in the glands and ecdysteroid titres in the course of larval and pupal development has been studied (Sedlak *et al.*, 1983), and in *Locusta migratoria*, a positive correlation between prothoracic gland secretory activity and haemolymph ecdysteroid titre has been observed (Hirn *et al.*, 1979). Both light microscopic (Wolbert, 1979) and electron microscopic studies (Gersch *et al.*, 1975; Smith and Nijhout, 1982; Sedlak *et al.*, 1983) have confirmed the existence of a correlation between secretory activity and the morphology of the gland cell's peripheral channel system: the deeper the channels, the higher the secretory activity. In addition, morphological changes evidenced by the rate of cell growth in the prothoracic glands also provides indirect information about secretory activity (Okot-Kotber, 1982). These criteria have been used in this analysis in *Spodoptera* to determine gland activity during normal larval development, which was monitored with respect to

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age, body weight, behaviour, and morpho-physiological changes.

In another Lepidopteran, *Manduca sexta*, prothoracic gland activity is under brain control mediated by neuroendocrine, and possibly nervous activity (Gilbert *et al.*, 1981). Initially, to analyse the neural influence on gland activity and metamorphosis in *Spodoptera*, we have separated the brain and associated ganglia from the prothoracic glands at times throughout the last-larval instar.

Together, the experiments described here provide a developmental profile of glandular activity and resultant systemic hormonal changes before and during metamorphosis, which is a necessary prelude to elucidating the factors and mechanisms involved in the hormonal control of development.

MATERIALS AND METHODS

Culture and preparation of animals

The cotton leafworm, *Spodoptera littoralis*, was reared on the leaves of *Taraxacum officinale* under a 18L:6D photoregime at 29°C. Freshly moulted last (sixth)-instar larvae were kept individually in petri dishes. Under these conditions all larvae which ecdysed to the last instar at night stopped feeding after 48 h on the third night, and pupated on the fifth night, 96 h after moulting to the last instar (Zimowska, 1984). These animals which began metamorphosis during the third night were used for all experiments. In the course of their development both behavioural (feeding activity; wandering behaviour; and construction of the putation cell) and morpho-physiological (dorsal vessel exposure; gut purging and larval–pupal ecdysis) changes were observed. Normally, larvae were weighed twice a day at the beginning and at the end of the dark periods. Dorsal vessel exposure and gut purging, accompanied with loss of weight, were used as morphological criteria for the beginning of metamorphosis. Starting at the beginning of the second day after the last-larval moult, 30–35 larvae per group were weighed and ligated with nylon monofilament between the head and thorax at 3-h intervals throughout the phagoperiod, and the second half of the last-larval instar. The neck-ligated larvae were examined daily for pupal cuticle production. The ability of these animals to metamorphose was correlated to their age and weight at the time of ligation.

Morphological and cytological analysis

Prothoracic glands were dissected out from precisely timed last-instar larvae at various ages. The size of gland cells was determined in Ringer's solution by calculating their maximal cross-sectional surface area in a Thoma-Zeiss counting chamber under a measuring ocular. For each measurement six pairs of glands were taken from larvae of known age, with all visible cells analyzed (in total, no less than 400 gland cells per measurement). The glands for cytological observations were dissected out at 12-h intervals throughout the last-larval instar. The tissue was fixed in alcoholic Bouin's fluid and embedded in paraffin wax. Five μm thick sections were stained with Harris' haematoxylin–eosin.

Ecdysteroid radioimmunoassay and chromatography

Radioimmunoassay for ecdysteroids was performed as described previously (Borst and O'Connor, 1974; Handler, 1982) using [^{3}H]ecdysone radioligand (80 Ci/mmol; New England Nuclear) and antisera kindly provided by Dr J. D. O'Connor. The Horn I-2, 17-week antiserum (Horn *et al.*, 1976) was used for most assays, with DHS I-15 antiserum (Soumoff *et al.*, 1981) used for some samples including all thin-layer chromatography (TLC) analyses. These antisera have ecdysone to 20-hydroxy ecdysone binding ratios of 2:1 and 5:1, respectively. Despite the different binding ratios, the few samples assayed with both antisera did not show appreciable differences in ecdysteroid titre. Haemolymph from individual larvae (75–300 μl) was diluted to 1 ml 70% methanol/10^{-3} M phenylthiourea for each sample, and duplicate aliquots of 50 and 100 μl taken from these methanol extracts were assayed. Standard curves were performed for each assay using unlabelled 20-hydroxy ecdysone, and therefore all titres are 20-hydroxy ecdysone equivalents which are expressed as "ecdysteroids".

Thin-layer chromatography was performed as described previously (Handler, 1982). Methanol extracts of haemolymph from 2 to 4 animals were dried under nitrogen and resuspended in 0.1 ml methanol before chromatography. Samples were streaked on a TLC silica gel plate (F-254, Merck Co.) and separation performed in chloroform–95% ethanol (4:1). RIA of 0.75 cm TLC fractions extracted in 1 ml methanol was performed with DHS I-15 antiserum using both ecdysone and 20-hydroxy ecdysone standard curves.

RESULTS

There are two distinct phases in the last-larval instar development of *Spodoptera littoralis*. The first one is a time of feeding (phagoperiod) lasting 2 days, and the second one is the period when the larva prepares itself for metamorphosis. At the end of the second 48-h period, larval–pupal ecdysis occurs. At specific times during this 4 day period different types of behavioural and morphophysiological changes were observed in the larvae which were correlated to changes in size, general morphology, and cytology of the prothoracic glands, as well as with changes in haemolymph ecdysteroid titres. At several times previous to and after the initiation of metamorphosis, chromatographic analysis of these ecdysteroids was performed.

In addition, sets of larvae were neck-ligated at various times to determine the influence of the brain and closely associated ganglia on the ability, and the time taken for these animals to metamorphose. To control for possible leakage of head factors, heads were removed in about 50% of the animals subsequent to ligation. The ability of these neck-ligated animals to metamorphose and the "quality" of metamorphosis was correlated to the critical conditions of larval age and weight, attained at the time of ligation. This experiment should suggest when the prothoracic glands have received sufficient information from the brain to effect metamorphosis.

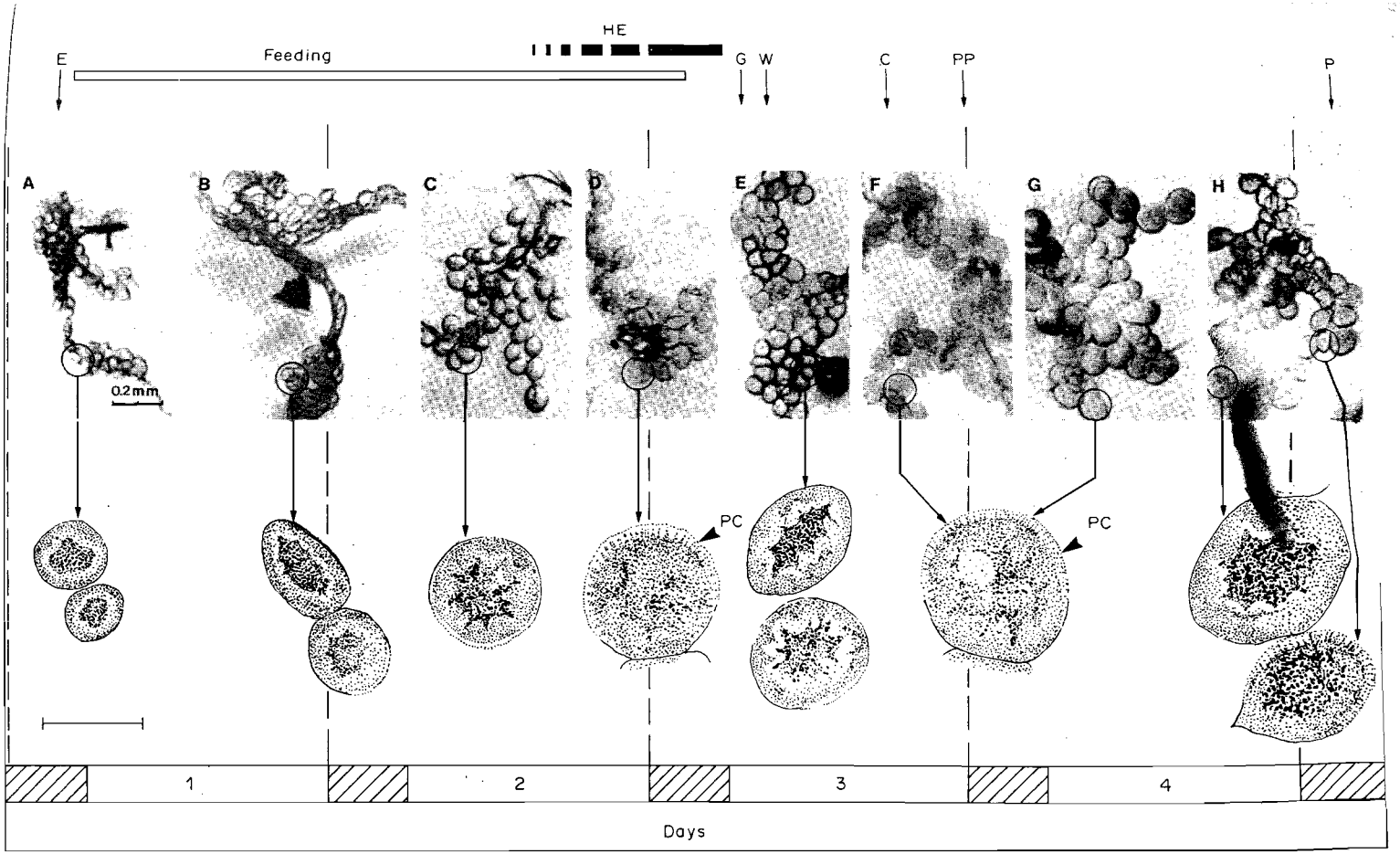

Fig. 1. Morphological and cytological changes in the prothoracic glands during the course of the last-larval instar. Encircled cells represent the region of the prothoracic gland and general external morphology of gland cells whose cytology is represented below. Cytology is represented by drawings of 5 μm sections of cells examined under a compound light microscope (scale line = 80 μm). The size of major structures (e.g. cell membrane; nucleus; cytoplasm; radial channels) were measured with an ocular micrometer and drawn to scale. The glands were dissected out from the last instar larvae at (A) 0–2, (B) 24, (C) 36, (D) 48, (E) 54, (F) 72 (G) 86, and (H) 96 h after the final larval ecdysis. E, ecdysis to the last larval instar; HE, heart exposure; G, gut purge; W, wandering behaviour; C, construction of the pupation cells; PP, prepupa; P, larval–pupal ecdysis; PC, peripheral channels. The dark periods of each day (6 h) are designated by closed bars, while the light periods (18 h) are designated by open bars.

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Each pair of glands was found to be a relatively compact trilobed gland, consisting of approximately 60 polyploid cells. The gland cells of freshly moulted last-instar larvae were small (2×10^{-3} mm^2 in cross-section surface area) and rather rounded (Figs 1a, 2). During the first 24 h of the phagoperiod, at the initiation of feeding, none of the prothoracic gland cells showed any sign of secretory activity. The cells consisted of giant nuclei surrounded by a thin double layer of cytoplasm which stained deeper with cytoplasmic dyes at the surface of the cells. The nuclei had a relatively uniform surface (Fig. 1a).

The haemolymph ecdysteroid titres in these freshly moulted larvae were relatively low, in a range close to 45 ng/ml haemolymph (Fig. 3). Larvae neck-ligated within 24 h after the last-larval moult died without initiating metamorphosis (Fig. 4). After 24 h of feeding the prothoracic gland cells became larger (3.3×10^{-3} mm^2), and ovoid in shape (Fig. 2). The cytoplasm was slightly denser and stained deeper with cytoplasmic dyes, while the nuclei developed a less regular surface (Fig. 1b). During this time ecdysteroid titres dropped to a level of 7–13 ng/ml haemolymph (Fig. 3).

On the second day of the last-larval instar further growth of the gland cells was observed, initially among the terminal cells. By 36 h into the last instar a thin layer of radial channels appeared on the cell surface of some cells. This peripheral channel system appeared initially in the terminal prothoracic gland cells and in regions where there was no close contact between the cells. At the end of the second day (shortly before cessation of feeding), the size of the gland cells rached 4.7×10^{-3} mm^2 (Fig. 2). The nuclei became lobulated, and the cytoplasm was more homogeneous with distinctly lighter regions limited to the area adjacent to the cell membranes. Both of these morphological features have been considered to be distinct signs of secretory activity (Mala *et al.*, 1974). At 12 h before cessation of feeding the ecdysteroid titre average 21 ng/ml haemolymph (Fig. 3). Approximately 14% of the larvae neck-ligated at this time pupated, although pupation occurred about 10 days after ligation (Fig. 4).

Three hours before the third night of the last-larval instar, the ecdysteroid titre increased to 53 ng/ml haemolymph and about 80% of the larvae neck-ligated at this time pupated after 8.5 days (Figs 3, 4). At this time the ecdysteroid titre varied greatly among individual larvae, ranging between 4 and 100 ng/ml haemolymph. This variation may be due to a rapid increase in ecdysteroids near this time. At this time both ecdysone and 20-hydroxy ecdysone are present, although the prohormone is predominant (Table 1).

During the third night at the end of the phagoperiod all the prothoracic gland cells increased in size to about 6.4×10^{-3} mm^2 (Fig. 1d). The nuclear membranes were not visible, and the peripheral channels became very deep. At this time ecdysteroids increased to an approximate level of 110 ng/ml haemolymph (Fig. 3). Larvae finished feeding and the first signs of metamorphosis, including gut purge and a drop in body weight, were observed. This occurred as ecdysteroid titres ranged between 100–160 ng/ml haemolymph. Neck-ligation performed at the onset of the third night resulted in 100% pupation after 5 days (Fig. 4). By the beginning, and at the end of the third night these ecdysteroids were almost exclusively 20-hydroxy ecdysone (Table 1).

In the first half of the third day after cessation of feeding, larval body weight decreased dramatically. At this time the gut purge occurred and prothoracic gland cells decreased in size to 4.4×10^{-3} mm^2 (Fig. 2). The nuclei of some of the cells became visible again, and the peripheral channel system appeared very shallow, although most of the cells still showed signs of activity (Fig. 1e). At this time the percentage

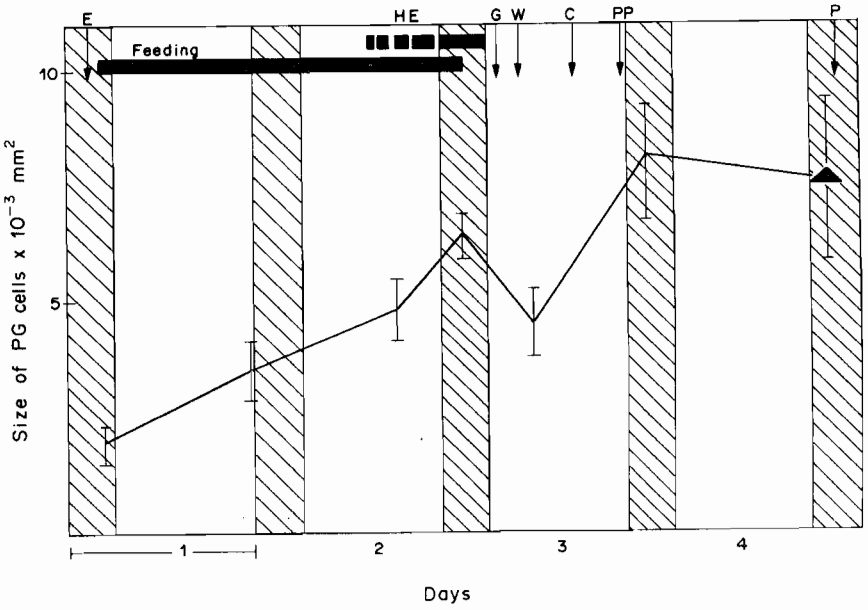

Fig. 2. Changes in size of the prothoracic gland (PG) cells during the last-larval instar. Size plotted as mean value ± SD. Further explanations given in Fig. 1.

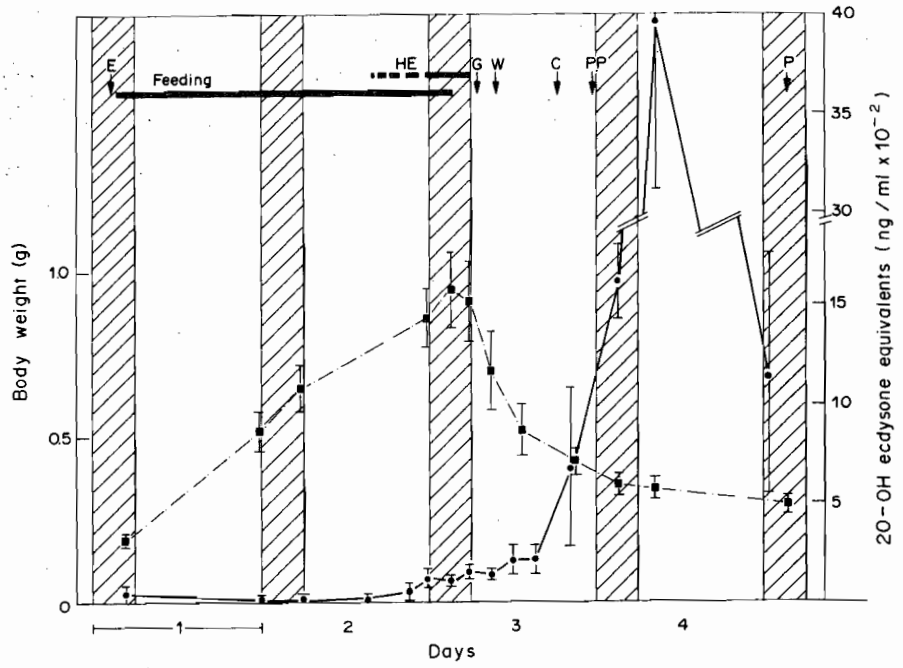

Fig. 3. Changes in body weight (■–·–■), and haemolymph ecdysteroid titres (●——●) during the last-larval instar. At designated time points, groups of larvae (120 animals) were weighed, and haemolymph extracted from three to four animals for individual RIA analysis. Values plotted as the mean ± SD from four separate assays.

of neck-ligated larvae able to pupate dropped to about 90%, while the remaining 10% reached the prepupal stage but never synthesized pupal cuticle (Fig. 4). The ecdysteroid level ranged between 140–210 ng/ml haemolymph (Fig. 3).

During the second half of the third day the rate of decrease in body weight was slower, and slightly shortened larvae started construction of their pupation cell. At the end of the third day all the prothoracic gland cells became highly active, as determined by their size (8.2×10^{-3} mm^2); deep peripheral channels; and lobulated nuclei (Figs 1f, 2). All the larvae ligated

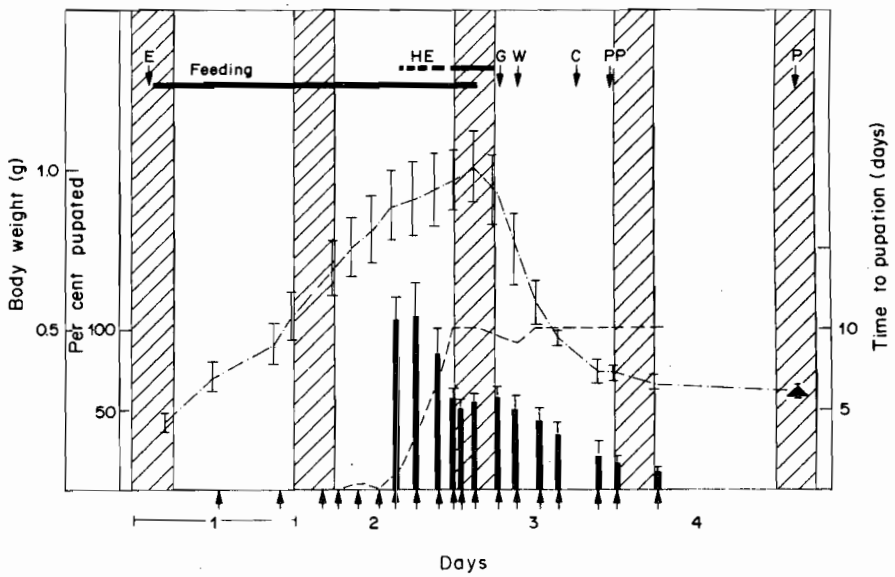

Fig. 4. Period of development and metamorphosis of larvae neck-ligated at different times during the last-larval instar. Times of ligation (↑); body weight at the time of ligation (–·–·–·–); per cent of pupations (– – – –); and the histogram (columns) indicates the time elapsed from ligation until pupation. Further explanations given in Fig. 1.

Table 1. Molar ratios of ecdysteroids during last-larval instar development

Time in development	Ecdysone:20-hydroxy ecdysone*
3 h before third night	1.7:1
Onset of third night	1:38
End of third night (time of switch-over)	1:33
3 h before fourth night	1:11
3 h after fourth night	1:1

*Determinations made by TLC of haemolymph extracts of 2-to-4 timed animals, as assayed by RIA using standard curves to ecdysone or 20-hydroxy ecdysone. Significant detection of polar or apolar products is noted in the text.

at this time pupated in less than 5 days (Fig. 4). Three hours before the end of the third day, ecdysteroid titres averaged about 700 ng/ml haemolymph, ranging between 300–1200 ng/ml (Fig. 3). Ninety per cent of these ecdysteroids were 20-hydroxy ecdysone, with the remaining material consisting of ecdysone and a small amount of more apolar substances (Table 1). At this time larvae completed construction of their pupation cells and became immobile.

During the first half of the fourth day there was a rapid increase in the rate of rising ecdysteroid titres, reaching a maximal level of about 4000 ng/ml haemolymph. Larval–pupal ecdysis occurred at the end of this day and during the fifth night. Prothoracic gland cell size remained quite large (7.8×10^{-3} mm^2) a few hours before the larval–pupal ecdysis, and in freshly ecdysed pupae. In some of these cells peripheral channels were still deep, indicating continued secretory activity (Figs 1g, 1h).

In freshly ecdysed pupae the ecdysteroid titre dropped considerably, although to the relatively high level of approx 1140 ng/ml haemolymph. The terminal prothoracic gland cells became very large (Fig. 1h), and nuclei were granulated and dense. The cell membranes were completely smooth indicating a post-secretory cellular state.

DISCUSSION

Prothoracic gland structure and ecdysteroid titres

The secretory activity of prothoracic gland cells during the last-larval instar of *Spodoptera littoralis* can be divided into four separate phases (Fig. 5). During the first half of the phagoperiod the initial phase occurs, and may be referred to as the "inactive phase". While the presence of ecdysteroids was noted (about 45 ng/ml haemolymph) in freshly moulted last-instar larvae, gland cell structure did not show any signs of activity. Unlike *Calpodes* (Dean *et al.*, 1980), freshly moulted last-instar *Spodoptera* larvae apparently have residual ecdysteroids in their haemolymph, possibly from the prothoracic gland activity cycle during the previous instar. During the second scotophase the gland cells were small and remained inactive, and haemolymph ecdysteroid titres decreased to a low level (7–13 ng/ml haemolymph).

The second phase. A spontaneous prothoracic gland activity phase occurred during the second half of phagoperiod. The terminal gland cells increased in size with some of them developing radial channels at

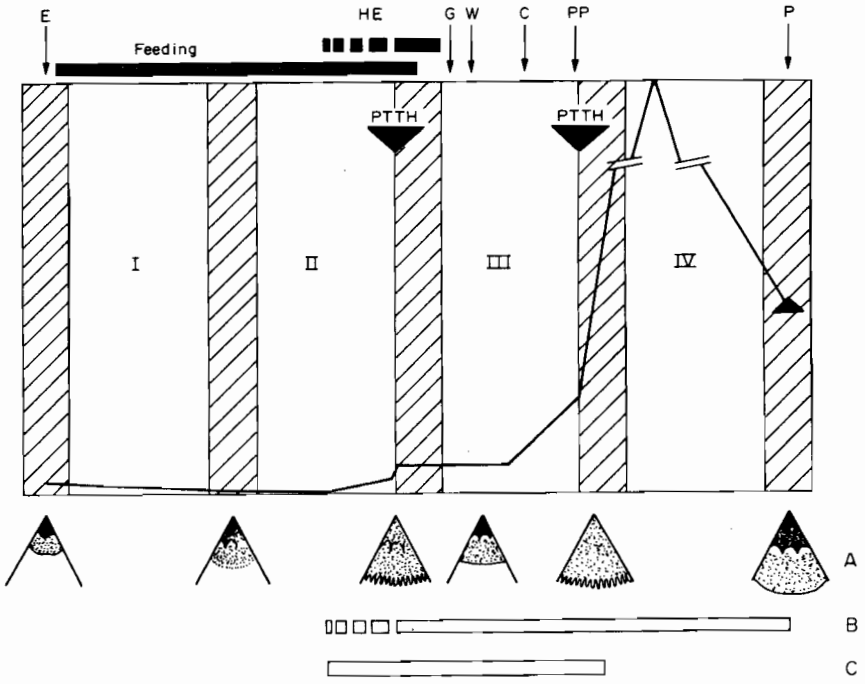

Fig. 5. Summary of the cellular events in prothoracic gland cells accompanied by changes in ecdysteroid titres. PTTH—presumptive time of the prothoracicotropic hormone release. A, schematic diagram of the changes in prothoracic gland cells during the last-larval instar; B, times when neck-ligated larvae developed to the larval–pupal ecdysis (continuous column indicates metamorphosis of all headless larvae); C, times when neck-ligation delayed the larval–pupal ecdysis as compared to non-ligated control larvae. Prothoracic gland activity is divided into four phases: I, inactivity; II, spontaneous activity; III, moderate activity; IV, full activity. Further explanations given in Fig. 1.

their surface, which has previously been considered to be a sign of secretory activity (Mala *et al.*, 1974; Wolbert, 1979). At this time the ecdysteroid level slowly increased, and by the end of the second day of the instar, reached a level of 53 ng/ml haemolymph.

The third phase. A phase of moderate activity began on the third night of the last-larval instar and lasted for 24 h. During the third night all the prothoracic gland cells were large and possessed very deep peripheral channels. Recently Sedlak *et al.* (1983) has suggested that deep peripheral channels occur as a result of a prothoracicotropic hormone effect on prothoracic gland cells. It is clear that at this time the glands are active, indicated by ecdysteroid titres increasing to a level of 110 ng/ml haemolymph.

At the beginning of the third day there was a small decrease in gland cell size and many of the cells did not show secretory activity, although ecdysteroid titres stayed at the same level. In the second half of the third day ecdysteroid titres increased to 700 ng/ml haemolymph, while prothoracic gland cellular structure indicated a moderate secretory activity in these glands.

The fourth phase. A full activity phase occurred at the end of the last-larval instar, at the onset of the fourth night. Cytological examination indicated that all the prothoracic gland cells were very active, supported by ecdysteroid titres increasing to a level of 1600 ng/ml haemolymph. The maximal titre of ecdysteroids occurred on the fourth day, just prior to the larval–pupal ecdysis.

These results indicate that the changes in prothoracic gland secretory activity are limited to the night, which is consistent with the possibility that a transition from light to darkness provides a synchronizing cue for gland activity. This might be true for *Samia cynthia ricini* as well, in which ecdysteroid titres first arise during the night (Fujishita *et al.*, 1982).

Regulation of the prothoracic gland secretory activity

Experiments with other lepidopterans, as well as *Spodoptera*, indicate at least two major hormonal factors regulating prothoracic gland activity. Prothoracicotropic hormone produced by brain neurosecretory cells and released by the corpus allatum in *Manduca sexta* (Gilbert *et al.*, 1981), serves to activate ecdysone secretion from the prothoracic glands. The action of another factor, juvenile hormone, on the gland activity is apparently more complicated. High juvenile hormone titres inhibit prothoracic gland activity early in the last-larval instar, while late in the instar juvenile hormone influence is reversed, resulting in activation of prothoracic gland function (Hiruma and Agui, 1982; Cymborowski and Zimowska, 1984).

The ligation experiments performed throughout the last-larval instar of *Spodoptera* allowed us to establish the head-critical period for prothoracic gland activity. Since headless larvae are able to metamorphose, their glands can, at some times, synthesize and liberate ecdysone without the immediate action of head factors. Larvae neck-ligated during the first 36 h of the phagoperiod did not metamorphose possibly indicating that at this time either a lack of nutrients, a lack of neural activation, or the possible maintenance of a high level of juvenile hormone inhibited the secretory activity of the prothoracic glands. While a lack of nutrients may be responsible for metamorphosis not occurring during the first 24 h (Zimowska, 1984), a high juvenile hormone titre may be more directly responsible since, in *Manduca sexta*, starvation results in the maintenance of high juvenile hormone titres due to decreased juvenile hormone esterase levels (Cymborowski *et al.*, 1981). A high level of juvenile hormone at the times of ligation is possible since two peaks of haemolymph juvenile hormone, at the beginning and at the end of the last-larval instar, have been observed in *Spodoptera litura* (Yagi, 1976), and in other Lepidopteran larvae (Varjas *et al.*, 1976; Peferoen and De Loof, 1979).

In *S. littoralis*, 12 h before cessation of feeding, 14% of the neck-ligated larvae metamorphosed. At this time a rise in haemolymph ecdysteroid titres to about 21 ng/ml began, and the percentage of neck-ligated larvae able to metamorphose increased as the ecdysteroid titre increased with time. Therefore it was impossible to discover a precise critical time for metamorphosis in *Spodoptera littoralis*, as was done for *Calpodes ethlius* (Dean *et al.*, 1980). In *Calpodes*, a precise time was discovered before which none of the ligated larvae pupated, while after this time 100% of them metamorphosed. In *Spodoptera* we have shown cytological evidence for the prothoracic glands secreting ecdysone before cessation of feeding.

In the second half of the second day, neck-ligation resulted in metamorphosis after an extended period of 8.5–10 days, due to a delay in the ecdysteroid peak (Cymborowski and Zimowska, 1984). This is persuasive evidence that the prothoracic glands can function without continuous stimulation from the head.

Ligatures made at the onset of the third night 3 h later, resulted in distinct quantitative and qualitative differences in the subsequent metamorphosis of these headless larvae, compared to the previous stage. All the neck-ligated larvae pupated about twice as rapidly, relative to previously ligated larvae. At this time, the first increase in ecdysteroid titres above low basal levels was noted. In *Manduca sexta*, the first surge of prothoracicotropic hormone (Truman and Riddiford, 1974) correlates to the first small peak of ecdysteroids in the haemolymph (Bollenbacher *et al.*, 1975), with a further confirmation of this relationship obtained by *in vitro* studies (Gilbert *et al.*, 1981). Nevertheless, there is an apparent species specificity concerning the critical conditions necessary for prothoracic gland activation by head factors (Lafont *et al.*, 1977; Mala *et al.*, 1977; Fujishita and Ishizaki, 1982). In *Spodoptera*, release of prothoracicotropic hormone at the beginning of the third night is suggested, although this remains to be proven (see Fig. 5).

Several hours after ecdysteroid titres initially elevate to more than 110 mg/ml haemolymph, general prothoracic gland morphology and cytology indicate a lack of cellular secretory activity. This possibly explains why approx 15% of the larvae neck-ligated at this time did not metamorphose, reaching only a prepupal stage. This inactive period occurs at the onset of the third day, but there is no resultant drop in ecdysteroid titre, thereby allowing most of the

neck-ligated larvae to successfully pupate. The factor inhibiting prothoracic gland activity is not known at this time.

The long period of development required by larvae neck-ligated late in the third day, compared with the unligated controls, indicates the involvement of a head factor in regulation of the gland activity. After the fourth night of the last instar, when an abrupt increase in ecdysteroid titre occurs, neck-ligation did not delay metamorphosis. By this time the prothoracic glands are certainly activated by head factors (prothoracicotropic hormone and/or juvenile hormone) leading to the large peak of haemolymph ecdysteroids prior to pupation.

Since the onset of both the small and large increases in ecdysteroid titres are limited to the beginning of night (the light to dark transition), a possible activation of these increases by prothoracicotropic hormone may be under photoperiodic regulation. For 3 h before this presumptive hormone release there were great variations in ecdysteroid titre, whereas 3 h later a synchronous and abrupt rise in ecdysteroids was observed.

The role of ecdysteroids in the last-larval instar

Our results indicate a correlation between morpho-physiological and behavioural changes during the last-larval instar, and changes in a haemolymph ecdysteroid titres (Fig. 5). The first elevation of ecdysteroid titre (approx 110 ng/ml haemolymph) occurs 3 h before cessation of feeding, and about 6 h before gut purge and the subsequent onset of wandering behaviour. In *Manduca sexta*, wandering behaviour follows a small peak of ecdysteroids (approx 70 ng/ml haemolymph) which occurs in the middle of the last-larval instar (Bollenbacher *et al.*, 1981). In *Mamestra brassicae*, the gut purge and wandering behaviour are initiated 24 h after a small peak of ecdysteroids (approx 30 ng/ml haemolymph) appearing on the third day of the last-larval instar (Agui and Hiruma, 1982).

The results of this study show that the first distinct rise in ecdysteroid titre occurs during the scotophase (dark period), followed by morpho-physiological (dorsal vessel exposure) and behavioural (gut purge and wandering) changes. Similar results were also obtained with *Samia cynthia ricini* (Fujishita *et al.*, 1982) where injection of 20-hydroxy ecdysone accelerated the gut purge, suggesting hormonal control of this behaviour.

In *Spodoptera littoralis*, the drop in body weight is consistently accompanied by gut purging, making these events reliable markers for the onset of metamorphosis which occurs when the ecdysteroid titre reaches a plateau of 140–210 ng/ml haemolymph. When the ecdysteroid titre increases to 700 ng/ml haemolymph, the larvae then build their pupation cells. This peak titre can be compared with the peak of ecdysteroids found in the larvae of *Galleria mellonella* (Sehnal *et al.*, 1981) and *Ephestia kuehniella* (Giebultowicz *et al.*, 1981), but for these animals it is not known whether the rise in ecdysteroid titre is the cause, or the result of the spinning behaviour.

A relatively large surge of ecdysteroids before pupation has been found in all holometabolous larvae investigated thus far (Delbecque *et al.*, 1975; Calvez *et al.*, 1976). In *Spodoptera*, this peak of about 4000 ng/ml haemolymph occurs 18 h before the larval–pupal ecdysis, and is about two to three times higher compared to other Lepidopteran species. In *Manduca sexta* for example, this peak reaches a level of 1500 ng/ml haemolymph, (Bollenbacher *et al.*, 1981), or 1300 ng/ml haemolymph in *Mamestra brassicae* (Agui and Hiruma, 1982). The same relative difference applies to the first small peak of ecdysteroids. These quantitative differences may be due to the relatively high level of ecdysone at these times (Bollenbacher *et al.*, 1981), and the use of antisera with different ecdysone-binding affinities, resulting in varying ecdysteroid titres. Nevertheless, the qualitative profiles of ecdysteroid fluctuations remain quite comparable during development among these lepidopterans.

Thin-layer chromatography analyses indicated that 2-hydroxy ecdysone or the ecdysone prohormone were indeed the major ecdysteroids being measured by our RIA procedure, with a relatively low proportion of apolar material observed at some times. The importance of the ecdysone to 20-hydroxy ecdysone proportion at specific times is difficult to assess, though certainly hormone conversion and metabolism are also important to the hormonal regulation of development.

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